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**BLOOD CONCENTRATION OF MARBOFLOXACIN AND ITS IN VIVO EFFECT IN YELLOW-BELLIED SLIDER TURTLES (TRACHEMYS SCRIPTA SCRIPTA), AFTER A SINGLE INTRACOELOMIC INJECTION AT THREE DOSE RATES**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1585870> since 2018-04-05T19:23:13Z

*Published version:*

DOI:10.1053/j.jepm.2016.06.011

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# **Blood Concentration of Marbofloxacin and Its in vivo Effect in Yellow-Bellied Slider Turtles (*Trachemys scripta scripta*), After a Single Intracoelomic Injection at Three Dose Rates**

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## **ABSTRACT**

Marbofloxacin is a commonly used antibiotic drug to treat bacterial infections in pet animals. Similarly to other fluoroquinolones, marbofloxacin is subject to the emerging problem of antimicrobial resistance. Thus, it is necessary to use PK/PD integration and modeling to minimize the selection of resistant mutants of bacteria and extend the useful life of antimicrobial agents, including marbofloxacin. This study evaluated the pharmacokinetics and the in vivo efficacy of marbofloxacin in *Trachemys scripta scripta* after a single intracoelomic injection. Three groups of 8 healthy adult turtles were each treated with 0.4, 2 and 10 mg/kg of marbofloxacin, respectively. Blood samples and rectal swabs were collected to measure marbofloxacin concentrations in serum (using an HPLC-FL validated method) and to evaluate in vitro antimicrobial susceptibility (using Kirby-Bauer Method), respectively. The PK profiles of marbofloxacin fit a bi-compartmental model and were dose dependent. The T<sub>max</sub> and half life ranged between 2.82-4.64 hours and 16.14-30.68 hours, respectively. Bacteria isolation showed the presence of both *E. coli* and *Salmonella* spp. *Salmonella* spp. was isolated only 48 hours after the administration of marbofloxacin and all strains were considered resistant. Results seem to suggest that even if marbofloxacin is able to reach considerable plasma concentrations, it can still be ineffective against bacteria that are potential animal and human pathogens.

**Key words:** Marbofloxacin; pharmacokinetics; in vivo effect; turtles; antimicrobial resistance

## **INTRODUCTION**

With the increasing popularity of herpetoculture, the treatment of bacterial infections in these animals should be based on a rational scientific approach. The most common pharmacokinetic/pharmacodynamic (PK/PD) approach for anti-microbial agents uses plasma concentration as the pharmacokinetic input value and minimum inhibitory concentration (MIC) as the pharmacodynamic input value.<sup>1</sup> These are necessary to establish efficacious therapeutic treatment modalities that can be augmented by a better understanding of these variables for the intended species.<sup>1</sup> Marbofloxacin (M) is an antimicrobial agent belonging to the class of fluoroquinolones developed exclusively for veterinary use and differs from other fluoroquinolones because of its oxadiazine cycle. Marbofloxacin has a wide spectrum of bactericidal activity, mainly covering gram-negative pathogens including *Pseudomonas* spp., but it is also effective against some gram-positive pathogens such as *Staphylococcus* spp., and *Mycoplasma* spp.<sup>2</sup> The proven efficacy and the valid pharmacodynamic and pharmacokinetic characteristics (i.e.: rapid bactericidal activity, wide spectrum of activity, large volume of distribution, low plasma protein binding, and relatively low minimal inhibitory concentrations (MICs) against target microorganisms) demonstrated in small and large mammals,<sup>3</sup> has

resulted in M commonly being used in an extra-label capacity for different animal species including reptiles.<sup>4</sup> Significant inaccuracies may result when effects and consequences of drugs for the species of interest are predicted based on extrapolation from other species which have marked differences in their xenobiotic metabolism and physiological processes.<sup>5</sup> Obtaining data on treatment options is also important as chelonian species are becoming increasingly popular as pets (i.e.: in Europe and the USA). The last few decades have seen an increased interest in exotic animals, particularly reptiles and amphibians, as domestic pets. An estimated 1% of households in the USA and Europe are currently raising an exotic animal, and of these, turtles are the most commonly owned.<sup>6</sup> Approximately 640,000 live reptiles are annually traded.<sup>7</sup> Despite continuing efforts to regulate this global trade of live animals, illegal importation of wildlife species flourishes in developed countries and tortoises and semi-aquatic turtles are the most frequently purchased.<sup>8</sup> Due to their rich and diverse microbiological biota, especially their propensity to act as carriers of *Salmonella* spp., these animals and their environments are potential reservoirs of infection, and are thought to have been responsible for numerous human salmonellosis outbreaks. A recent study suggests that annually, 74,000 cases of human salmonellosis in the United States are linked to reptile and amphibian exposure.<sup>9</sup> These data are strictly connected to the emerging problem of antimicrobial resistance that involved several antibiotic drugs, including fluoroquinolones.<sup>1,10</sup> Aside from the public health issues, these animals are considered as pets and are treated as members of the family, it follows that pet owners demand the same level of care they expect for themselves.<sup>11</sup> The availability of specific, accurate, and targeted treatments is taken foregranted. Additionally, these animals represent a food source in some countries and for this reason; there is a need to determine the effects of xenobiotics entering the food chain through these animals. This is particularly important as reptiles are increasingly being used as a human food source (e.g. alligator meat, snakes, turtles, and turtle eggs). Considering all the aforementioned factors, the aim of the present study was to provide PK/PD data after single intracoelomic injection of M at 3 dose rates in yellow-bellied slider turtles (*Trachemys scripta scripta*).

## **MATERIAL AND METHODS**

### **Animal Treatment and Sampling**

Twenty-four clinically normal turtles (*Trachemys scripta scripta*) of both sexes (4 males, 20 females), with body weights ranging from 0.40 to 2.3 kg (mean 1.1 kg), supplied by a local park, were used for the study. The turtles were acclimated for a 2-week period prior to commencement of the study. Turtles were judged to be in good health based on physical examination at the time of acquisition and at the start of the study, and through daily observation of behaviour and appetite. These observations were made by specialized veterinary personnel (M.S.). Animal care and handling was performed according to the provision of the EC council Directive 2010/63 and also according to Institutional Animal Care and Use directives issued by the Animal Welfare Committee of the University of Pisa, which approved the study protocol. Turtles were equally assigned to three treatment groups (A, B and C, each n=8) using 24 slips of paper marked with the numbers 1 to 24 in a box, according to a randomized, open, single-dose, three-treatment, one period, parallel study design. The proportions of sexes in each group were 2 males in group A and 1 male in group B and C. The 3 groups were placed in different 300 L plastic pools, with water depth of 25 cm, water temperature of 25 °C, and custom-built mechanical and biological filtration. A dry basking area was heated to 30 °C using an infrared lamp. Ambient temperature in the room varied from 24 to 26 °C (electronic temperature sensors assured the constant temperature in both the water and basket area). Turtles were fed with a floating pelleted diet three times per week. Prior to use, injectable M (Marbocyl 1%, Vetoquinol, UK) was diluted with saline in order to inject the same volume of drug per kg of animal, in the three groups. This was to reduce dosing errors due to the bias in small volume injections. Marbofloxacin was administered as a 0.4, 2, and 10 mg/kg bolus by intracoelomic injection in the left prefemoral fossa using a sterile 22-gauge 3.75 cm needle to group A, B, and C, respectively. Blood samples (0.5 mL and 0.25 mL in subjects over or below 0.5 kg body weight, respectively) were collected from the dorsal cervical,

subcarapacial venipuncture site at 0, 0.5, 4, 10, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h after M administration. Although blood collected from the subcarapacial site can become contaminated by lymph, this was not considered an issue as fluoroquinolones have been reported to be equally distributed in blood and lymph as a result of their wide volume of distribution,<sup>12</sup> the pharmacokinetic data obtained should not have been affected. Blood was immediately transferred to tubes containing citric acid (Microtainer; Becton Dickinson and Company, Franklin Lakes, NJ USA), vortexed and stored at -20 °C until use within 30 days from collection. Sterile swabs were gently inserted in the first cloacal tract of each turtle daily (0, 24, 48, 72, 96, 120, 144, 168, 192, 216, and 240 h) after M administration. Swabs were immediately stored in sterile cryotubes (Nunc®, Sigma Aldrich, St. Louis, MO USA) at -80 °C until the analyses were performed.

### **Chemical and Reagents**

Pure M and enrofloxacin (internal standard [IS]) powders (> 99.0% purity) were purchased from Sigma-Aldrich (St. Louis, MO USA). High-performance liquid chromatography (HPLC) grade acetonitrile (ACN), dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>) and ethylacetate (CH<sub>3</sub>COOC<sub>2</sub>H<sub>5</sub>) were purchased from Merck (Darmstadt, Germany). Tetraethyl amine was obtained from Sigma Chemical Co. (St Louis, USA). Phosphoric acid, sodium dihydrogenophosphate, disodium hydrogenophosphate heptahydrate and potassium dihydrogenophosphate were purchased from Carlo Erba (Milan, Italy). Deionised water was produced by a Milli-Q Millipore Water System (Millipore, MA USA). All the other reagents and materials were of analytical grade and supplied from commercial sources. The aqueous and organic components of the mobile phase, degassed under pressure, were mixed by the HPLC. The LC mobile phases were filtered through 0.2 µm cellulose acetate membrane filters (Sartorius Stedim Biotech, Aubagne Cedex) with a solvent filtration apparatus. For bacterial isolation and identification, the following culture media were used: Xylose Lysine Deoxycholate Agar with Novobiocin (XLD + N); Muller Kauffmann Tetrathionate broth with Novobiocin (MKTTn); Brilliant Green Agar (BGA); Triple Sugar Iron (TSI); Mueller-Hinton Agar (MH), these were purchased from Liofilchem (Roseto degli Abruzzi, Teramo, Italy). Marbofloxacin-impregnated disks for the Kirby Bauer method were obtained courtesy of ATI s.r.l. (Ozzano Emilia, Bologna, Italy).

### **Preparation of Solutions**

Singular stock solutions of M and IS in water were prepared using dark volumetric flasks at an individual concentration of 1,000 µg/mL, and subsequently stored at +4 °C. To reach the final concentration (100 µg/mL), appropriate dilutions of stock standard solutions were prepared diluting 1 mL of each solution to 10 mL. The solution of M was then diluted in dark glass tubes (10 mL) to reach final concentrations of 10, 5 and 1 µg/mL. These were stored at +4 °C. This latter concentration (1 µg/mL) was then diluted to prepare a five-point calibration curve of the analytes at the following concentrations: 0.200, 0.100, 0.050, 0.025, and 0.010 µg/mL. The M solution was stable for at least 10 weeks if stored at +4 °C.

### **Instrumentation and Chromatographic Conditions**

The HPLC system was an LC system (Jasco, Japan) consisting of a high pressure mixer pump (model PU 980 Plus), spectrofluorometric detector (model 2020 Plus) and a loop of 50 µL. Data was processed using Borwin software (Jasco, Inc.). Chromatographic separation assay, modified from Giorgi et al. (2013) was performed by a Sinergy polar RP 80A analytical column (150 x 4.6 mm inner diameter, 4 µm particle size, Phenomenex, Torrance, CA USA) maintained at 25 °C. The mobile phase consisted of ACN: aqueous solution (20:80 v/v%) at a flow rate of 1 mL/min. The aqueous solution consisted of potassium dihydrogenphosphate (0.02M), phosphoric acid (0.006M) and tetraethyl amine (0.012M) in water. Excitation and emission wavelengths were set at 295 and 500 nm, respectively.

### **Sample Extraction**

The procedure was based on that previously reported by Giorgi, et al<sup>12</sup>, with slight modifications. Briefly, aliquots (0.2 mL) of blood were added to 0.1 mL of IS (500 ng/mL) and diluted with 800  $\mu$ L of 0.1M phosphate buffer at pH 7.4. After adding 6 mL of a mixture of CH<sub>2</sub>Cl<sub>2</sub>, the samples were shaken at 200 oscillations/minutes, for 10 minutes and centrifuged at 4,000 g for 5 minutes. Five mL of the organic layer was transferred into a clean tube and dried at 40 °C under nitrogen stream. The residue was dissolved in 0.2 mL mobile phase, vortexed and an aliquot was injected into the chromatographic system.

### **Quantification**

Standard curves were constructed with standard M concentrations vs. ratio of M/internal standard peak areas. Linearity of the regression curve was assessed on the basis of the residual plot, the fit test and the back calculation (within 20% of known amount). The quantitative HPLC method was validated by examining the measurement of consistency of results (within-run and between-run), correlation (coefficient of determination of the standard curve), and extraction efficiency of the assay. The within-run precision was calculated from similar responses from six repeats of three control samples (10, 100, and 200 ng/mL) in one run. The between-run precision was determined by comparing the calculated response (in ng/mL backfit of the standard curve) of the low (10 ng/mL), middle (100 ng/mL), and high (200 ng/mL) control samples over three consecutive daily runs (total of six runs). The assay accuracy for within-run and between-run was established by determining the ratio of calculated response to expected response for low (10 ng/mL), middle (100 ng/mL), and high (200 ng/mL) control samples over six runs. The extraction efficiency was determined by comparing the response (in area) of low, middle, and high standards, and the internal standard, spiked into blank plasma eluent before evaporation, to the response from equivalent extracted standards.

### **Bacteria Isolation and marbofloxacin Efficacy**

Samples were examined with a four-step procedure: pre-enrichment, enrichment, selective and differential culture media and identification. The swabs with cloacal or faecal materials were subjected to pre-enrichment; each sample was soaked in 9 mL of buffered peptone water (BPW) medium for 30 s, and then stored at -20 °C. The BPW was incubated at 37 $\pm$  1 °C for 18  $\pm$  2 h. The enrichment step used 100  $\pm$  10 mL of BPW dispersed in 10  $\pm$  0.5 mL of Müller Kauffmann tetrathionate broth with novobiocin (MKTn), this was incubated at 37  $\pm$  2 °C for 24  $\pm$  3 h. One mL of colony material from the opaque zone of the colonies was streaked on Xylose Lysine Deoxycholate agar with novobiocin (XLD + N) and Brilliant Green Agar (BGA) using a sterile disposable loop, and incubated at 37  $\pm$  2 °C for 24  $\pm$  2 h. Colonies suspected to be *Salmonella* spp. on the XLD + N and/or BGA plates were inoculated into Triple Sugar Iron (TSI) and incubated at 37 °C for 24 h. Identification was performed using a commercial micromethod (API 20 E; BioMerieux, Rome, Italy).

### **In vitro Antimicrobial Susceptibility Test**

Each strain was analyzed to evaluate the antimicrobial susceptibility following the Kirby Bauer method (Bauer et al., 1966) described in Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>13</sup> Using a sterile inoculating loop or needle, material was collected from four or five isolated colonies of organisms; the colonies were then suspended in 2 mL of sterile saline solution and the tube was vortexed to create a smooth suspension (adjusting the turbidity to 0.5 McFarland standards). A sterile swab was dipped into the inoculum solution and rotated against the side of the tube using firm pressure, to remove the excess fluid. Mueller-Hinton Agar was inoculated by streaking the swab three times over the entire dried surface of the agar. Appropriate antimicrobial-impregnated disks were placed on the surface of the agar, using forceps to dispense each antimicrobial disk one at a time. Then, all plates were incubated at 37 °C for 18 hours. Kirby-Bauer test protocol provides for the measurement of inhibition zone of microbial growth around the disk. Using a caliper, each zone was measured with the unaided eye while viewing the back of Petri dish. In particular, the pharmacological activity of M against different bacteria was tested. On the recording sheet,

depending on the inhibition zone size, the strain was classified as susceptible (S;  $> 18$  mm), intermediate (I;  $14 < X < 18$  mm), or resistant (R;  $< 14$  mm), compared to the reference values.

### **Statistical Analysis and Pharmacokinetic Evaluation**

The animal sample size was determined for a study with a 80% power, a  $P < 0.05$  and a standard deviation of about 30% as averaged by former similar studies in turtles.<sup>12,14</sup> Marbofloxacin plasma concentration versus time curves were modeled for each subject using a mono- or a two-compartment open model with different weighing factors (1,  $1/c$ ,  $1/c^2$ ).<sup>15</sup> Comparison between competing models was made using the random distribution residuals, visual inspection of the goodness of fit curves and the Akaike's information criterion. The pharmacokinetic calculations were carried out using WinNonlin v 5.3.1 (Pharsight Corp, Sunnyvale, CA, USA). The results obtained from the Kirby-Bauer isolation test were organized and analysed using Prism 5.0 software (Graph Pad). Normality was evaluated using Shapiro-Wilk test and data were elaborated using One-way Anova with Dunnet post test, assuming a significant value at  $P < 0.05$ .

## **RESULTS**

### **Animals**

Two days after the treatment, 2 turtles belonging to the highest dose group died. Apart from this, other animals did not demonstrate visible local or systemic adverse effects. Until the time of an animals death, blood samples and cloacal swabs were collected as required by study protocol.

### **Pharmacokinetics**

The PK profiles of M, fitted by a bi-compartmental model, were dose dependent, showing  $C_{max}$  and AUC values increasing proportionally with the increasing dose (Table 1). The ranges of  $T_{max}$  and half life were quite variable, 2.82-4.64 hours and 16.14-30.68 hours, respectively. Marbofloxacin was quantifiable in the three treatment groups from the first collection time (0.5 hours) to 144 hours although not in all the animals. The three averaged curves were parallel up to 96 hours, then the 0.4 mg/kg group deviated. The plasma concentration versus time curves are reported in Figure 1.

### **Kirby-Bauer Isolation Test**

*Escherichia coli* was isolated in all the groups at T0. The mean of the zone of inhibition against *E. coli* was  $35.4 \pm 0.6$  mm,  $36.0 \pm 0.9$  mm, and  $36.5 \pm 0.9$  mm, for Groups A, B, and C, respectively. According to the CLSI guidelines, data indicated the bacteria were sensitive to M. No bacteria were isolated in any of the groups at T24 hours. Beginning at T48 hours, strains of *Salmonella* spp. were identified with all of these bacterial isolates having an inhibition zone close to 0 mm, indicating a strong resistance to M. In group B only, two strains of *E. coli* resistant to M were isolated at T 96 h. Figure 2, 3, and 4 represent the results concerning the bacterial isolation in different groups at all time points. Figure 5 summarizes the total amount of each species of bacteria isolated during the present study: the majority of the isolated bacteria (26/36) were *Salmonella* spp. resistant to M. Figure 6 shows the comparison between the bacteria isolated at each experimental time point and for each treatment group. One way Anova highlighted a statistically significant difference ( $P < 0.05$ ) was observed between group A at T96 hours versus the other groups and experimental time points. Group A at this time point demonstrated the largest amount of bacteria, all of which were resistant to M.

## **DISCUSSION**

The aim of the present study was to evaluate the pharmacokinetics of M in yellow-bellied slider turtles (*Trachemys scripta scripta*) and to integrate these data with pharmacodynamic parameters concerning M's influence on populations of cloacal bacteria. The PK data obtained in the present study established that intracoelomic injection at 0.4, 2, and 10 mg/kg were all detectable 5 minutes after the drug administration.

Drug was detectable in each single animal at different ranges of time, showing a large intergroup variability. The large variability in pharmacokinetic values in wild animals has been previously reported.<sup>12,14</sup> However, it might have been exacerbated by the randomized grouping of the animals that did not consider the sex, weight, and age of the test subject. At 120 hours after the drug administration, animals in group A showed an increase in plasma concentration. Several factors might have contributed to this unexpected result: i) the small number (n=6) of animals showing quantifiable concentrations of M close to the LOD of the method; ii) damage to the water filtration/recycling system of the pool where the animals were placed; iii) other undocumented factors. The calculation of AUC and C<sub>max</sub> demonstrated concentration dependent behavior, according to the increasing dose administered to different groups. This was also demonstrated in previous research.<sup>16</sup> Considering the route of administration, some authors claimed that oral treatment is the most suitable route of administration in turtles.<sup>17,18</sup> However, oral administration requires either pharyngostomy tube placement or potentially uncomfortable head restraint. These two options can cause pain to the animals and, the latter technique in particular, can be stressful to the animal, especially in small turtles. Some studies involving fluoroquinolones injected via the intramuscular route in reptiles, reported that local irritation and soft tissue necrosis can occur through this route of administration.<sup>17-20</sup> It has been recently reported that intracelomic administrations of diluted fluoroquinolones have not shown the above mentioned adverse effects. No localized adverse side effects were observed in the present study. For these reasons, in the present study the intracelomic route was used as it was considered more reproducible and suitable in terms of animal welfare. The pharmacokinetic analysis showed that the C<sub>max</sub> at the clinical dosing (2 mg/kg) was slightly higher and drastically lower than those reported in mammals<sup>21</sup> and in *Caretta caretta* turtles<sup>2</sup> respectively, after IM administrations. Considering turtle species are more closely related to one another than to other orders of animals, this wide variation underlies the importance of conducting pharmacokinetic studies for individual species rather than extrapolating or transposing doses from data generated in other species.<sup>22</sup> Two turtles of the highest dose group died two days after M administration. Samples were collected up to the time of death and did not demonstrate any particular abnormalities. Only physical examination was performed and no necropsy evaluation was done on these subject turtles. In the authors' opinion, it is not possible to assume that the death of these animals was triggered by the high dose administration of M. Indeed, Shan et al.<sup>16</sup> reported that when a dosage of 10 mg/kg was administered, both orally and intramuscularly, in soft shelled turtles, the only side effects noted were transient and uncommon. The same dose regimen was also administered to cattle and similarly in this study, no toxic or lethal effects were seen.<sup>3</sup> The dose of 10 mg/kg of M was administered subcutaneously in koalas (*Phascolarctos cinereus*), and no toxic effects were reported although there was an increase in creatine kinase levels in blood samples collected after the administration.<sup>23</sup> Nevertheless, further, more directed studies should be performed to evaluate potential toxicity at such a high dosage, especially in chelonian species. The data obtained from bacterial isolation showed that at T<sub>0</sub>, only *E. coli* colonies were identified. *Escherichia coli* is normally present in cloacal or fecal samples, all of these were sensitive to the effect of M. In the authors' opinion, no other bacteria were isolated because the burden of *E. coli* was very high, and they would overgrow any other bacteria. After a short period where no bacteria were isolated, 48 hours after M administration, *Salmonella* spp. was isolated from all groups. Likely, these data suggest a good sensitivity to the M of *E. coli* normally present in cloaca. *Escherichia coli*, competes, as is known, with other bacteria for growth and living spaces at the level of the enteric microbiome. However, in this study, it is clear that the selective pressure of M eliminated *E. coli*, thereby enabling the growth and selection of M resistant *Salmonella* spp. From their initial isolation, these bacteria demonstrated a strong resistance to M. This resistance was maintained for all the subsequent time points until the end of the experiment. The experimental time point of T<sub>96</sub> h in Group A demonstrated a statistically significant difference ( $P < 0.05$ ) vs. all the other time points: the elevated number of isolates and the global resistance of the isolated bacteria seemed to suggest that a low concentration of M could be well tolerated by common bacteria, leading to the development of antibiotic resistance. These results are in line with a previous study on dogs showing that M was not able to eradicate *Staphylococcus intermedius* in Beagle dogs,<sup>21</sup> but conflicting with a



recent study performed in soft shelled turtles,<sup>16</sup> where M was demonstrated to have a bactericidal/bacteriostatic action against *Aeromonas hydrophila*. To the best of authors' knowledge, no other studies have evaluated *E. coli* and *Salmonella* spp. in vivo, in turtles nor in other animal species. Only the article by Andraud, et al<sup>24</sup> evaluated the antimicrobial effects of M using a pharmacodynamic in vitro model using swine fecal samples. The authors calculated several parameters and confirmed that the behavior of M is concentration-dependent, but the killing effect significantly depends on duration of exposure.<sup>24</sup> This last result partially supports the results of the present study, in which *E. coli* was isolated only at the first time point in all groups, and disappeared after drug administration. The difference between the two studies was that in Group A (treated with 0.2 mg/mL) in the present study, at 96 hours, two colonies of *E. coli* were isolated and were resistant to M. The development of antimicrobial resistance in recent decades has led to an intensification of discussion about the prudent use of antimicrobial agents, both in veterinary and human medicine. The consumption of several antimicrobials, extensively used in animal husbandry, was higher in animals than in humans, while consumption of antimicrobials critically important (CIA) for human medicine (e.g., fluoroquinolones, 3rd and 4th generation cephalosporins) was higher in humans.<sup>10</sup> In both humans and animals, positive associations between consumption of antimicrobials and the corresponding resistance in bacteria were observed in most cases. Although in vitro susceptibility testing remains the cornerstone of clinical antibacterial therapy, not all naturally occurring mechanisms of resistance can be detected by standard laboratory methodologies. At the end of the '80s, Sanders et al.<sup>25</sup> reported that bacterial resistance during cephalosporin treatment developed in some 20–40% of systemic infections with Gram-negative bacilli, accounting for treatment failure or relapse in at least 10% of such cases. In those years, hospital antibiograms reported 75–90% of *E. cloacae* and *S. marcescens* isolates to be susceptible to third-generation cephalosporins. In the next years, the same pathogens may acquire an antibiotic resistance, leading to an increase of treatment failure. It seems clear that without accurate laboratory detection and investigation regarding occult resistance phenotypes, the treatment of Gram-negative infections may remain suboptimal.<sup>26</sup> The long lasting pharmacokinetics of M in turtles, highlighted in previous studies and confirmed in the present paper, has other ramifications. In many Eastern cultures, turtles are part of the human food chain.<sup>27</sup> Consumption of turtle meats containing antibiotic residues could facilitate the rapid development of resistant bacteria in humans. This situation has been already emphasised in the “classical” food producing animals where the antimicrobial agents such as 3rd- and 4th-generation cephalosporins, fluoroquinolones, and macrolides are widely prescribed (accounted for 0.2%, 1.7% and 8%, respectively, of the total sales of drugs in 26 countries of European Union.<sup>28</sup> Hence it is important to study the pharmacokinetics and pharmacodynamics of antibiotics in each animal species. It is well known that dose or efficacy extrapolation from other animal species can lead to shortcomings. This is particularly true when the extrapolation occurs between species with marked differences in xenobiotic metabolism and physiological processes (i.e. mammals vs. reptiles).

## **CONCLUSION**

In conclusion, even if M is able to reach quickly detectable concentrations in turtle plasma, the efficacy against bacteria isolated in cloacal swabs was not optimal. Moreover, the isolated bacteria demonstrated a strong resistance to M. The results of the present study seem to suggest that the use of M for therapeutic purposes should be carefully considered, not only taking into account the low efficacy but also for the risk to induce antibiotic resistance in bacteria that could be potentially pathogenic for humans.

## **ACKNOWLEDGEMENTS**

This work was equally supported by athenaeum funds (ex 60% University of Pisa and Fondi per la Farmacovigilanza Regione Piemonte). No external funding was used to support the preparation of manuscript. Authors acknowledge Dr. H. Owen, University of Queensland, Australia for the English and scientific editing of the manuscript.

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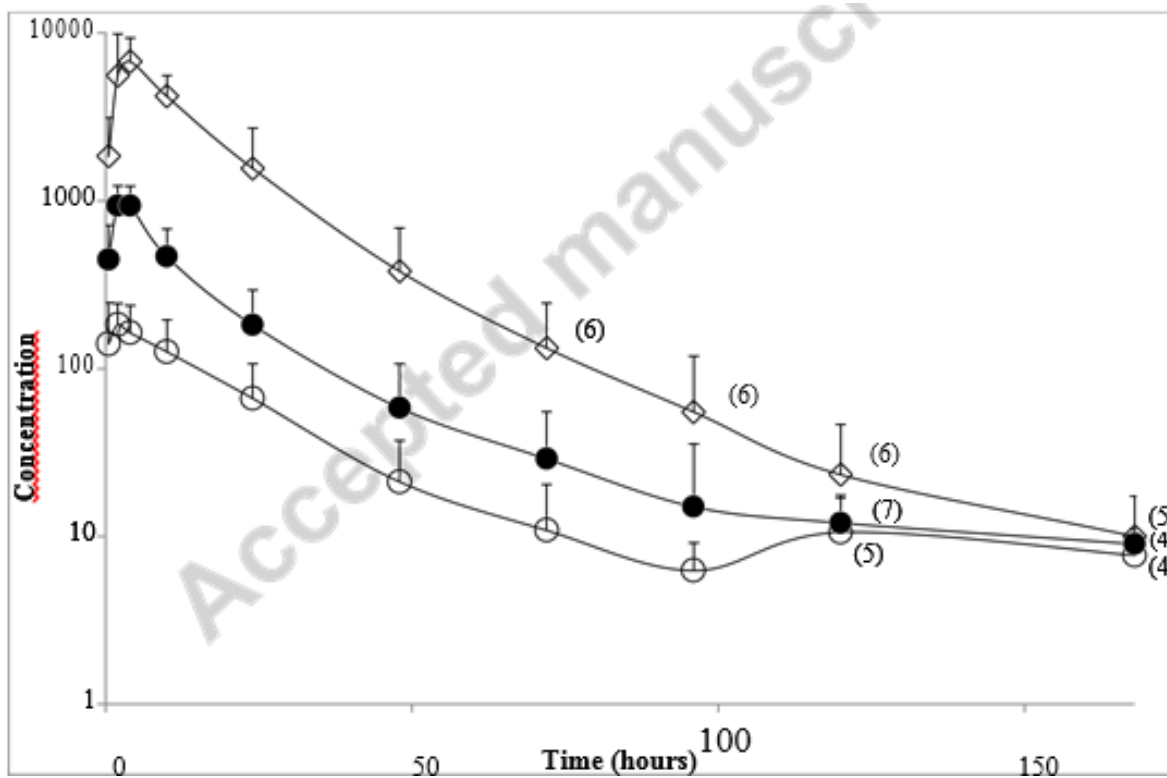
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Table 1

Main pharmacokinetic parameters after intracoelomic administrations of marbofloxacin at 0.4, 2 and 10 mg/kg in turtles (*Trachemys scripta scripta*).

Parameters	Units	Group I (0.4 mg/kg)			Group II (2 mg/kg)			Group III (10 mg/kg)		
		Mean	SD		Mean	SD		Mean	SD	
AUC <sub>0-∞</sub>	hr*ng/mL	4697.60	±	2770.84	16257.72	±	6066.75	139146.00	±	38097.94
K01 HL	hr	1.14	±	1.51	1.24	±	1.00	1.96	±	1.10
K10 HL	hr	10.48	±	5.58	6.90	±	3.74	8.84	±	4.79
Alpha HL	hr	6.42	±	4.64	3.14	±	1.13	3.73	±	2.51
Beta HL	hr	30.68	±	20.26	24.94	±	11.32	16.14	±	3.61
CL/F	mL/hr/kg	116.77	±	72.52	141.62	±	62.68	76.78	±	24.46
V/F	mL/kg	1590.37	±	1471.61	1525.01	±	1170.73	468.46	±	462.07
Tmax	hr	3.45	±	3.62	2.82	±	1.22	4.64	±	2.06
Cmax	ng/mL	235.36	±	44.03	1047.61	±	272.21	7145.83	±	3493.64
K01	1/hr	2.33	±	2.25	0.90	±	0.55	0.48	±	0.31
K10	1/hr	0.09	±	0.06	0.13	±	0.06	0.11	±	0.08
K12	1/hr	0.07	±	0.08	0.08	±	0.04	0.09	±	0.11
K21	1/hr	0.05	±	0.02	0.07	±	0.04	0.11	±	0.04



**Fig. 1.** Observed mean plasma concentrations of marbofloxacin after intracoelomic injection of 0.4 mg/kg ( $\circ$ , n=8), 2 mg/kg ( $\bullet$ , n=8) and 10 mg/kg ( $\diamond$ , n=8) in turtles (*Trachemys scripta scripta*). Bars represent standard error of the mean. Numbers in the brackets indicate the number of values used to calculate each mean value, if different from 8.

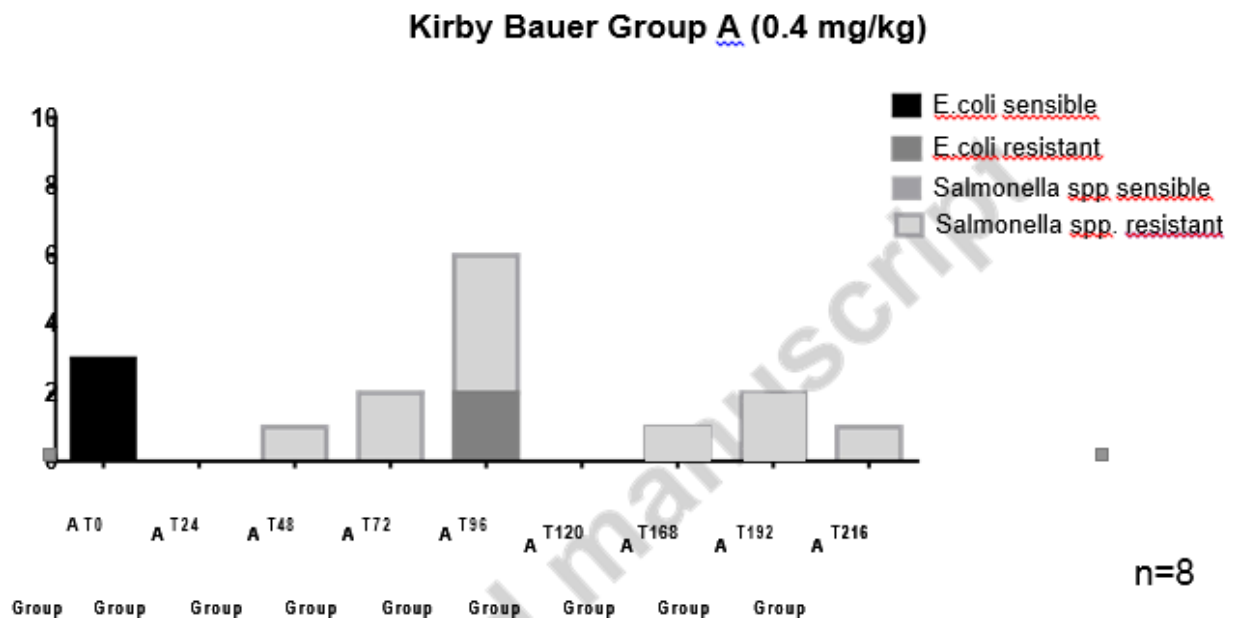


Fig.2: Distribution of isolated bacteria from cloacal swabs after single intracelomic administration of 0.4 mg/kg (Group A) of marbofloxacin in turtles (*Trachemis scripta scripta*). Bacterial sensitivity or resistance to marbofloxacin was test using Kirby - Bauer method (n=8).

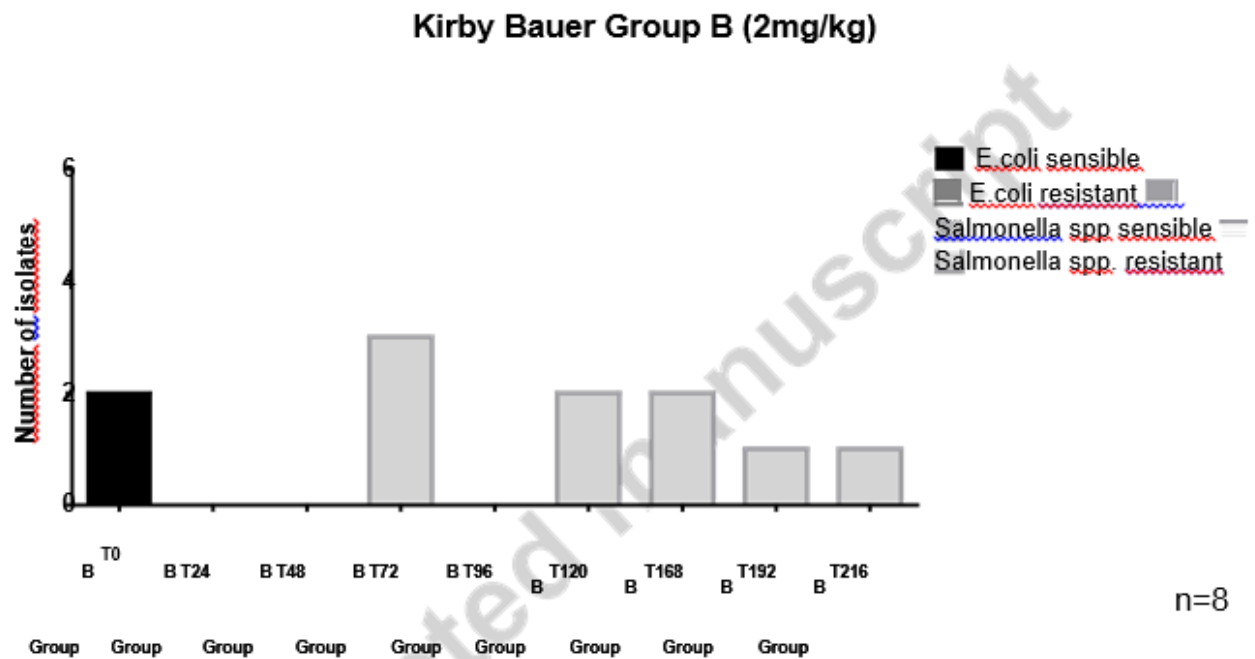
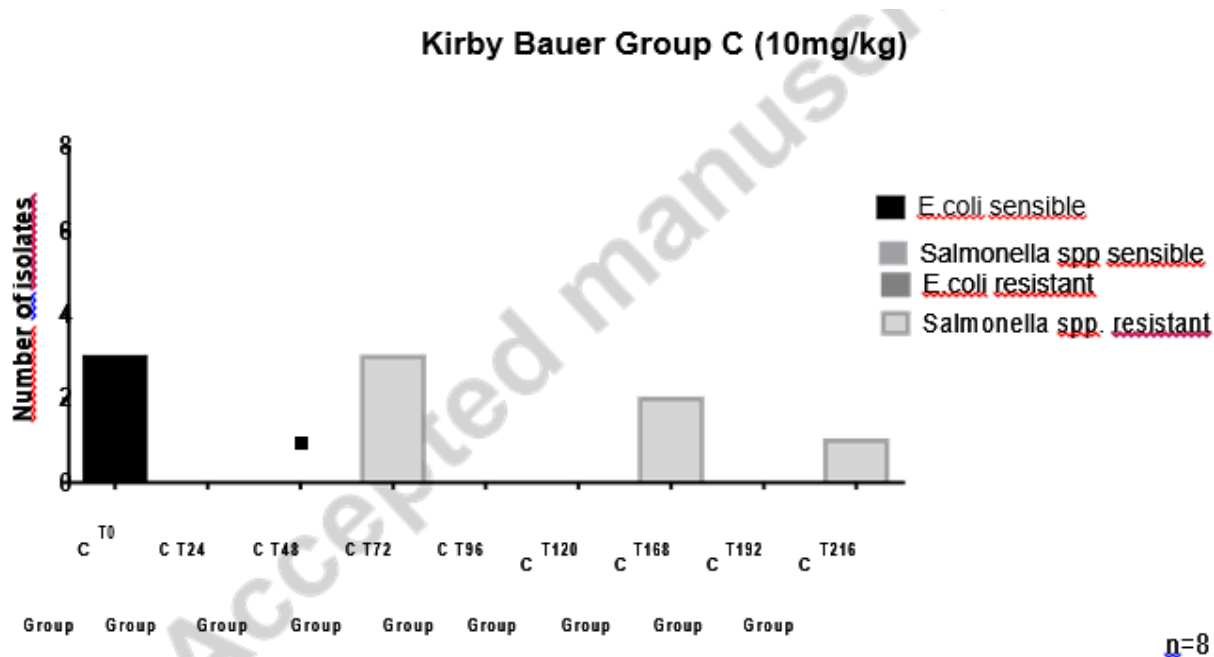


Fig.3: Distribution of isolated bacteria from cloacal swabs after single intracelomic administration of 2 mg/kg (Group B) of marbofloxacin in turtles (*Trachemis scripta scripta*). Bacterial sensitivity or resistance to marbofloxacin was test using Kirby - Bauer method (n=8).



**Fig.4:** Distribution of isolated bacteria from cloacal swabs after single intracelomic administration of 10 mg/kg (Group C) of marbofloxacin in turtles (*Trachemis scripta scripta*). Bacterial sensitivity or resistance to marbofloxacin was test using Kirby - Bauer method (n=8). The symbol ■ represents the death of two turtles 48 h after the drug administration: from that time point, n=6



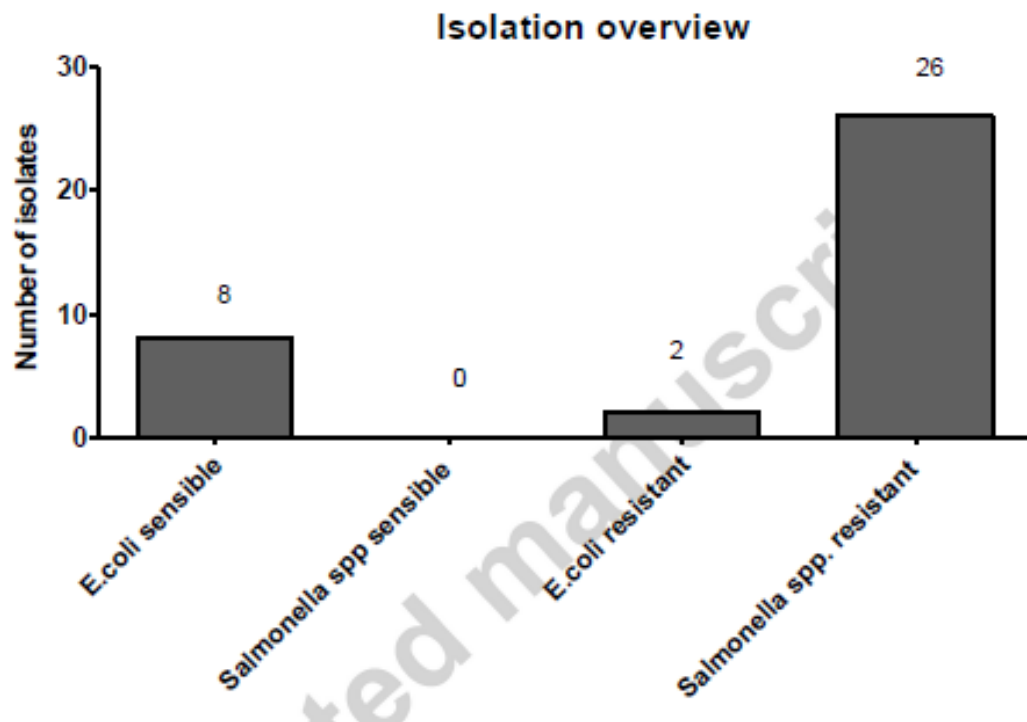


Fig.5: Total amount of each species of isolated bacteria and their sensitivity or resistancy to marbofloxacin using Kirby - Bauer method.

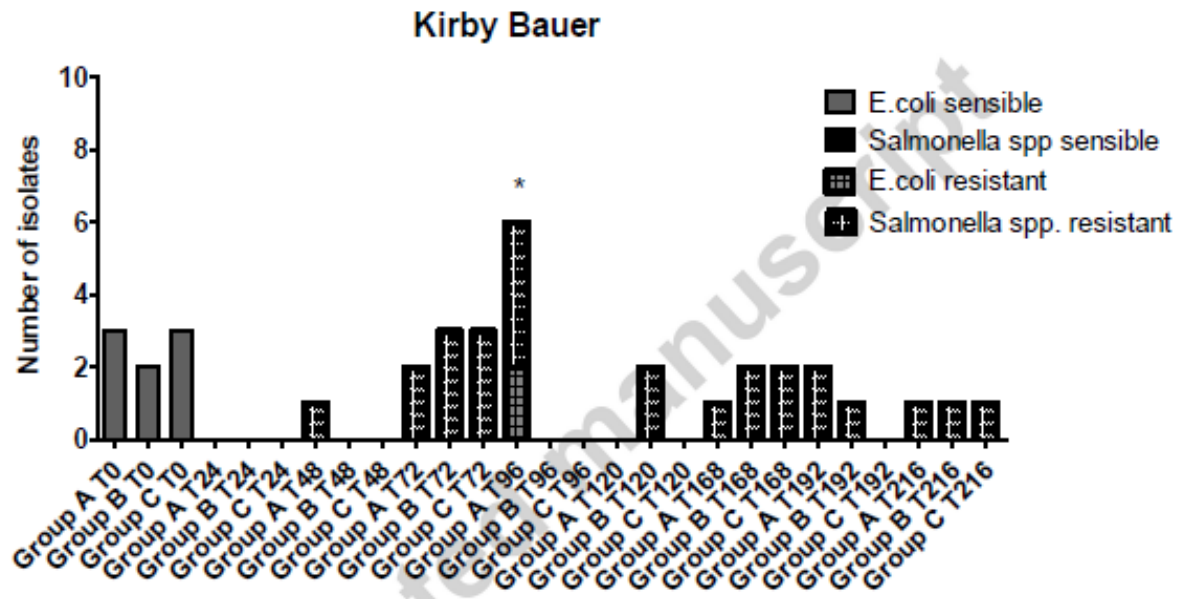


Fig.6: summary of all the isolated bacteria in each turtle group at each time point. Data were analysed using One way Anova with Dunnet post test ( $P < 0.05$ ). Only Group A at T96 h demonstrated a significant stastically difference vs.s the other time points (\*).